Plant Pathology (2005) **54**, 1–7 Doi: 10.1111/j.1365-3059.2004.01114.x

A combined agar-absorption and BIO-PCR assay for rapid, sensitive detection of *Xylella fastidiosa* in grape and citrus

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Application of the polymerase chain reaction (PCR) to disease diagnosis is limited in part by the presence of PCR inhibitors. Inhibition can be overcome and sensitivity increased by culturing bacteria on agar media prior to PCR (termed BIO-PCR). However, *Xylella fastidiosa* grows slowly, requiring 10–14 days for visible colonies to appear. In this study an agar-absorption BIO-PCR method for detecting *X. fastidiosa* in grape and citrus plants was developed. Optimum lengths of time for absorption of inhibitors by the agar medium or enrichment of bacteria on the medium were determined for Pierce's disease of grape and citrus variegated chlorosis. When petioles of grape and citrus leaves with symptoms were spotted onto agar media, the spots washed after various time intervals and assayed for *X. fastidiosa* by real-time PCR, 97% (31 out of 32) and 100% (six out of six) of spots were positive after 2 days and 4 h for grape and citrus, respectively. With direct PCR, only 12·5% (four out of 32) and 33% (two out of six) of spots were positive, respectively, and visible *X. fastidiosa* colonies were evident after 10 and 14 days, respectively. In a separate experiment with samples from a different vineyard, 46% (13 out of 28) of the grape samples (agar spots) were positive after 1 day and 93% (26 out of 28) after 5 days using agar-absorption PCR. In contrast, all samples were negative by direct PCR. Viable *X. fastidiosa* were recovered from all samples after 14 days. Further tests with eight randomly selected grape petioles from three Texas vineyards known to have Pierce's disease resulted in 50% being positive by a simple 24 h agar-absorption PCR assay, whereas none was positive by direct PCR. Overall, 10 out of 16 (63%) vines from five vineyards (two in California and three in Texas) were positive after the 24 h agar-absorption PCR assay. In contrast, only one vine was positive by direct PCR. This simple agar absorption-based PCR assay protocol should prove useful for the routine detection of *X. fastidiosa* and other slow-growing bacteria in the presence of PCR inhibitors.

Keywords: BIO-PCR, citrus variegated chlorosis, diagnosis, PCR inhibitors, Pierce's disease, real-time PCR

Introduction

Xylella fastidiosa (Wells *et al*., 1987), a Gram-negative, xylem-limited fastidious bacterium, causes diseases in many economically important plants in North and South America, including grape, citrus, coffee, peach, almond, plum, alfalfa, and several shade and landscape trees including elm, maple, mulberry, oak, sycamore and oleander (Hopkins & Adlerz, 1988; Hopkins, 1989; Rossetti *et al*., 1990; Sherald & Kosta, 1992; Opgenorth, 1995; Beretta *et al*., 1996). The diseases are all transmitted by sharpshooter insect vectors (Purcell, 1980; Brlansky *et al*., 1983). Heavy losses occur in grape when the vectors are abundant (Purcell, 1997). The introduction of the highly mobile glassy-winged sharpshooter *Homalodisca coagulata* into California has threatened the grape industry (Purcell & Saunders, 1999). Currently no means are available for controlling these diseases. Injection of diseased plants with antibiotics has been attempted, but with little success (Hopkins, 1989; Sherald & Lei, 1991). Control of insect vectors is very difficult, as the leafhoppers have many hosts and are active throughout the growing seasons. Presently, the only reliable methods are the use of pathogen-free planting material and the elimination of diseased plants. Any control programme for the management of these diseases should include reliable, sensitive methods for rapid diagnosis of infected plants. Several methods, cultural, serological and molecular, have been developed for this purpose (Davis *et al*., 1978, 1980; French *et al*., 1978; None *et al*., 1980; Hopkins, 1989; Sherald & Lei, 1991; Minsavage *et al*., 1994). Culturing is very slow and inconsistent, and requires pathogenicity testing which takes 1–2 months (Davis *et al*., 1978;

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Accepted 3 September 2004

Minsavage *et al*., 1994; Hopkins, 2001). Serological methods, including immunofluorescence (IF) and enzyme-linked immunosorbent assay (ELISA), are available, but are not very sensitive and cross-reactions often result in false-positive results (French *et al*., 1978; None *et al*., 1980). Amplification of pathogen-specific DNA sequences by classical polymerase chain reaction (PCR) has been used and is more sensitive than ELISA, but is much more time-consuming and requires additional technical training (Minsavage *et al*., 1994). A rapid, same-day, direct PCR technique is available for symptomless samples taken in early spring when the sap is flowing (Schaad *et al*., 2002). However, results of direct PCR are highly variable for samples taken in late summer when pathogen numbers are high and inhibitors are present in the plant sap (Rowhani *et al*., 1993; Minsavage *et al*., 1994; Botha *et al*., 2001). In attempts to develop a highly sensitive BIO-PCR technique (Schaad *et al*., 1995, 1999) and avoid inhibitors, preliminary results indicated that enrichment of the fastidious *X. fastidiosa* was not needed.

The present study reports for the first time the development of an agar absorption-based technique for elimination of PCR inhibitors for detecting *X. fastidiosa* in petioles of leaves. Using real-time PCR, results are obtained within 1–24 h after plating on agar media when the pathogen is present in high numbers, and within 5–7 days if the bacterium is present in low numbers and BIO-PCR (enrichment) is required.

Materials and methods

Comparison between direct PCR and agar-absorption PCR

Grape leaves with petioles were obtained from 1 July to 30 September 2001 from four different vines, 71-2, 93-1, 93-4 and 69-1 (row-plant number) showing symptoms of Pierce's disease in an infected vineyard in McFarland, CA, USA. The samples were sent overnight by commercial courier to Fort Detrick, MD, USA. Two petioles were sampled per vine. Each petiole was surface-sterilized separately by immersion in 1·05% sodium hypochlorite solution for 10 min, then rinsed twice (5 min each) in sterile distilled water. The petioles then were placed in a sterile plastic Petri dish and 5 mm sections cut off and discarded. Each petiole was cut into four equal segments numbered from one to four, starting at the stem end, resulting in a total of eight segments per vine. Each segment was cut in half. One half was squeezed with sterile forceps and the resulting droplet put into 1·5 mL microfuge tubes containing 50 μL sterile saline (0·85% NaCl). The tubes were vortexed, then centrifuged for 30 s at 10 000 ***g*** to collect the fluid. The samples were used immediately for direct real-time PCR or stored at –20°C. The other half of the segment was squeezed as above, and the end touched onto PD2 agar medium (Davis *et al*., 1980; Hopkins, 2001) at eight previously marked sites. One plate was used per petiole and the plates were incubated at 28°C. Each segment was coded in order to compare results from each half. One of the eight spots for each segment was removed with a sterile scalpel after 2, 3, 4, 5 and 6 days' incubation and used for PCR. Results were recorded as positive if fluorescence rose above the background level within 40 cycles and negative if it did not. The remaining three spots were kept for further incubation for visual confirmation. Any resulting growth was streaked onto PD2 agar for cloning and confirmation by real-time PCR.

Citrus samples, including leaves and twigs, were collected from a sweet orange tree (*Citrus sinensis* cv. Madame Vinous) infected by citrus variegated chlorosis (CVC) and maintained in the USDA-BSL-3 containment facility at Fort Detrick. The strain was originally from Sao Paulo, Brazil, and samples were prepared and analysed as described above for grape samples. A total of six individual leaf petiole samples with symptoms of CVC were used for this study.

Sampling from an agar medium

The uppermost layer of the medium within the marked site was removed aseptically with a sterile scalpel and placed in a 1·5 mL microcentrifuge tube containing 50 μL sterile molecular biology-grade (MBG) water, vortexed, and centrifuged briefly at 10 000 ***g*** to collect the fluid. The liquid was tested for the presence of *X. fastidiosa* by real-time fluorescent PCR as described below.

Determination of optimal time for sample recovery and BIO-PCR

Grape leaves and petioles were obtained from four vines with symptoms (17-13-1, 17-13-2, 17-100-1 and 30-100-1) from a second vineyard known to contain Pierce's disease in McFarland, as described above. Three petioles from each vine were surface-sterilized as above. After cutting 5 mm from both ends, a segment about 5 mm long was removed from each petiole and used for direct PCR, as above. From the remaining petiole material, six 1 cm segments (one for each incubation time) were cut. After squeezing with tweezers, the ends of each segment were touched to 14 marked sites on PD2 agar medium. One plate was used per segment. The 24 plates (six for each of the four vines) were incubated at 28°C. After 1 and 4 h, and 1, 3, 5 and 7 days' incubation, one plate of each vine was chosen randomly and the agar surface (2–3 mm) of every other spot (numbers 1, 3, 5, 7, 9, 11, 13) was removed and used for real-time PCR. Results were recorded as above. The remaining spots were used for visual observation and confirmation of the presence or absence of *X. fastidiosa* after 7–14 days, as above.

Effect of incubation time on quantitative recovery of *X. fastidiosa*

To determine the effect of incubation time on PD2 agar on the relative increase in numbers of *X. fastidiosa*, three additional petioles were used from vine 17-13-2. Direct PCR was performed on each petiole using 1 cm segments. Five segments (one per incubation time) were then cut from

the remaining petiole, and each squeezed and spotted onto a separate plate of PD2 agar medium at 35 previously marked sites. After 4 h and 1, 3, 5 and 7 days' incubation at 28°C, one plate was selected randomly, the agar surface (2–3 mm) of sites 1, 6, 11, 16, 21, 26 and 31 removed as described, and tested separately using real-time PCR. Results were recorded as cycle threshold (Ct) values (defined as the cycle number in which fluorescence rose above the background level) in order to determine any increase in growth of *X. fastidiosa*. The remaining 28 agar sites were used for visual observation and to confirm the presence or absence of *X. fastidiosa*. Any resulting growth was streaked onto PD2 agar for confirmation by real-time PCR.

Use of YDC agar medium for agar-absorption PCR control

It is well known that *X. fastidiosa* will not grow on general plating media (Hopkins, 2001). To determine whether cells of *X. fastidiosa* spotted onto yeast dextrose–$CaCO_3$ (YDC) agar medium (Wilson *et al*., 1967) resulted in a positive detection result without growth, one infected petiole from vine 17-13-2 was surface-sterilized as above and cut into three equal segments. Each segment was squeezed aseptically and spotted onto YDC agar medium at eight previously marked sites, and onto two sites on PD2 agar as a positive control. The YDC plates were incubated at 28°C, and after 4 h and 1, 3, 5 and 7 days' incubation the agar surface (2–3 mm) of appropriate spots was removed and tested by real-time PCR as described below. The YDC plates were incubated for 20 days to allow for any eventual growth of *X. fastidiosa* on the remaining three spots. The PD2 plate was kept for 20 days at 28°C.

Simplified 24 h agar-absorption assay protocol

To confirm the reliability, convenience and applicability of the agar-absorption PCR assay for rapid diagnosis of Pierce's disease, grape leaves and petioles showing scorch symptoms were collected in early September 2003 from three vineyards in Texas known to have Pierce's disease and sent overnight to Fort Detrick. Three leaves were selected from each of vineyards 1 and 2, and two were selected from vineyard 3. A 4–5 cm section of each petiole was surface-sterilized and rinsed, as described above. After discarding 1–2 mm from each end, 10 mm sections were cut off and soaked in 50 μL sterile water in a sterile microcentrifuge tube for 20 min. Samples of 5 μL were spotted onto four marked sites on PD2 agar and the plates incubated at 28°C. A 15 μL sample was stored at –20°C for direct PCR. After 24 h at 28°C, one spot was removed and treated as described above, and the washing fluid assayed by real-time PCR. The remaining three sites were used for visual observation after 14 days.

Real-time fluorescence PCR conditions

For routine tests, 1 μL aliquots were taken from each sample, added to 24 μL of a freshly prepared PCR master mix containing the following: 2·5 μL of 10 × PCR buffer without $MgCl_2$ (Applied Biosystems), 5·0 μL 25 mM $MgCl_2$, 2·0 μL 2·5 μM dNTPs (Invitrogen), 1·0 μL 25 μM forward primer (Invitrogen), 1·0 μL 25 μM reverse primer (Life Technologies), 0·5 μL 10 μM 5′ FAM, 3′ TAMRA-labelled (TaqMan, Applied Biosystems) *X. fastidiosa*-specific ITS probe (Schaad *et al*., 2002), 0·25 μL AmpliTaq DNA polymerase (5 units μL^{-1}) (Applied Biosystems), 5·0 μL 5 × Smart Cycler Additive Reagent (Cepheid), and 6·75 μL MBG water. All PCR reactions were performed in 25 μL tubes using a Smart Cycler TD system (Cepheid) and run as follows: preincubation at 95°C for 30 s, followed by 40 cycles at 95°C for 1 s and 62°C for 20 s (Schaad *et al*., 2002). For each PCR run, MBG water and 10–100 pg *X. fastidiosa* DNA (strain ATCC 35879) were used for negative and positive controls, respectively. All PCR samples were run in duplicate. In most cases the cycle threshold (Ct) values were not recorded, as quantitative differences between samples were not of interest here, only positive or negative results. The Ct values were recorded only for the experiment showing the effects of incubation time on the increase in growth of *X. fastidiosa* from grape petioles (Table 4).

Results

Comparison of direct PCR and agar-absorption PCR

When the squeezed grape petioles were touched onto PD2 agar medium, the agar turned brownish-red in colour. Four out of 32 (12·5%) and two out of six (33·3%) of grape and citrus samples, respectively, were positive by direct PCR (Tables 1 and 2). With the agar-absorption PCR, 31 out of 32 (Table 1) and six out of six (Table 2) samples were positive after 2 days' incubation for grape and 4 h for citrus, respectively. Visual observation of the PD2 plates after 10–14 days revealed the presence of small colonies growing on the three remaining spots, confirming the presence of *X. fastidiosa* in all the samples. All water controls were negative, and all the positive controls resulted in Ct values of 22–25.

Determination of optimal time for sample recovery and BIO-PCR

All naturally infected grape petioles were negative by direct real-time PCR and positive by BIO-PCR (Table 3). Observations of growth of *X. fastidiosa* on PD2 agar after 7–14 days showed the presence of tiny (0·2–0·4 mm) bacterial colonies, and identification by PCR confirmed the presence of *X. fastidiosa*. Results of agar-absorption PCR for vines 17-13-1 and 17-13-2 showed little difference between assays after 1 and 4 h, 1 and 3 days; most samples were positive. Agar samples of vines 17-100-1 and 30-100-1 were negative by PCR when removed and assayed after 1 h to 3 days' incubation. However, after 5 days' incubation, 11 out of 14 (79%) spots resulted in a positive PCR reaction. Two spots (numbers 3 and 13) from vine 30-100-1 remained negative by PCR for the

Table 1 Comparison between direct PCR and BIO-PCR for the detection of *Xylella fastidiosa* in naturally infected grape petioles of four vines from California

Vine[a]	Segment number[b]	Direct PCR[c]	Incubation time for real-time BIO-PCR[d] (days)		Visual[e]
			2	3–6	
71-2	1–8	–[f]	+[f]	+	10
93-1	1–8	–	+	+	10
93-4	1, 3–8	–	+	+	10
	2	–	–	+	
69-1	1,2,5,7	–	+	+	10
	3,4,6,8	+	+	+	
Controls[g]					
Negative		–	–	–	NA[f]
Positive		+	+	+	NA

[a]Leaf petioles from two leaves with symptoms per vine were used.
[b]Each petiole was cut in four equal parts (1 cm long), resulting in eight segments per vine.
[c]Each segment was cut in half. One half was used for sap extraction; 1 μL used for direct real-time PCR as described in the text.
[d]The other half of the same segment was squeezed and spotted onto PD2 agar at eight marked sites and incubated for 2–6 days. Agar from each of five of the eight sites was removed after 2, 3, 4, 5 and 6 days, added to water, vortexed, and 1 μL used for direct PCR as described in the text. The three remaining sites were kept for visual growth assessment.
[e]Days needed to observe growth of *X. fastidiosa* on the three remaining sites.
[f]–, Real-time PCR signal of probe (fluorescence) failed to rise above background within 40 cycles; +, signal rose above background within 40 cycles; NA, not applicable.
[g]Negative controls consisted of MBG water and positive controls consisted of *X. fastidiosa* DNA; controls were included in each PCR run.

Table 2 Effect of incubation time on PD2 agar on real-time BIO-PCR results for the detection of *Xylella fastidiosa* in naturally infected citrus leaves

Leaf[a]	Direct PCR[b]	Incubation time on PD2 agar and PCR results[c]		Visual[d]
		4 h	5, 7, 9 days	
1	–[e]	+[e]	+	14
2	+	+	+	14
3	+	+	+	14
4	–	+	+	14
5	–	+	+	14
6	–	+	+	14
Controls[f]				
Negative	–	–	–	NA[e]
Positive	+	+	+	NA

[a]Six segments (1 cm long) from each leaf petiole and mid-vein were cut and selected.
[b]Each segment was cut in half. One half was squeezed and soaked in 50 μL water, and 1 μL used for direct PCR as described in the text.
[c]The other half of the same segment was squeezed and spotted onto eight marked sites on PD2 agar. After 4 h and 5, 7 and 9 days, the agar surface of one site was removed with a scalpel and used for PCR.
[d]Days of incubation after which colonies of *X. fastidiosa* became visible by eye.
[e] –, No PCR signal (fluorescence); +, fluorescence rose above background within 40 cycles; NA, not applicable.
[f]Negative controls consisted of MBG water; positive controls consisted of DNA of *X. fastidiosa*; controls were included in each PCR run.

entire 7 days' incubation, and one spot (number 13) from vine 17-100-1 remained negative after 5 days' incubation, but became positive after 7 days' incubation (Table 3). For the four vines, 13 out of 28 (46%) spots were positive by agar-absorption PCR after 1 day and 25 out of 28 (93%) were positive after 5 days. All spots retained for visual growth observation were positive after 14 days. All water controls were negative, and all positive controls resulted in Ct values of 22–25.

Effect of incubation time on quantitative recovery of *X. fastidiosa*

All three petioles were negative by direct PCR. However, all were positive after a short 4 h agar-absorption treatment. There was little difference in recorded Ct values (relative numbers of cells of *X. fastidiosa*) after 4 h, 1 and 3 days (Table 4). However, Ct values dropped considerably after day 5 and decreased even more at day 7, showing an increase in cell numbers. Mean Ct values were 36·1, 34·2, 35·1, 29·2 and 26·8 at 4 h, day 1, day 3, day 5 and day 7, respectively (Table 4). All water controls were negative, and all positive controls were positive (Ct values of 22–25). In the case of citrus samples, Ct values started decreasing only after 7 days' incubation (data not shown), showing the slower growth of the citrus pathogen. All water controls were negative, and all positive controls resulted in Ct values of 22–25.

Use of YDC agar medium for agar-based PCR control

Xylella fastidiosa was detected from each of the petiole segments of vine 17-13-2 spotted onto YDC agar

Table 3 Determination of optimal time for sample recovery of *Xylella fastidiosa* in naturally infected grape petioles by real-time BIO-PCR

Vine	Number of petioles	Direct PCR[a]	Spot no.[b]	Incubation time for real-time BIO-PCR[c]						Visual[d]
				1 h	4 h	1 day	3 days	5 days	7 days	
17-13-1	3	–	1,3,5,9,13	+[e]	+	+	+	+	+	7
			7	–[e]	+	+	+	+	+	
			11	–	+	–	+	+	+	
17-13-2	3	–	1,3,5,9,11	+	+	+	+	+	+	7
			7	+	–	+	+	+	+	
			13	+	–	+	–	+	+	
17-100-1	3	–	1–11	–	–	+	–	+	+	12
			13	–	–	–	–	–	+	
30-100-1	3	–	1,9,11	–	–	–	–	+	+	14
			5,7	–	–	–		+	–	
			3,13	–	–	–	–	–	–	
Controls[f]										
Negative	NA[e]	–	NA	–	–	–	–	–	–	NA
Positive	NA	+	NA	+	+	+	+	+	+	NA

[a]Sap was extracted from a 1 cm section of each petiole; 1 μL was used for direct real-time PCR; –, all three samples negative.
[b]From the remaining petiole materials, six 1 cm long segments (one for each incubation time) were cut and selected. Each segment was squeezed and spotted onto a separate plate of PD2 agar medium at 14 previously marked sites. Plates were incubated at 28°C. Seven spots were used for PCR (odd numbers) and seven (even numbers) for visual assessment.
[c]Agar from odd-numbered segment spots was removed after 1 and 4 h, 1, 3, 5 and 7 days, added to water, vortexed, and 1 μL used for direct PCR as described in the text.
[d]Days needed to observe growth of *X. fastidiosa* on seven even-numbered spots 2–14.
[e]+, Signal of probe (fluorescence) rose above background (baseline) within 40 cycles; –, signal failed to rise above background within 40 cycles; NA, not applicable.
[f]Negative controls consisted of MBG water, positive controls DNA of *X. fastidiosa*; controls were included in each PCR run.

Table 4 Effect of incubation time on the increase in numbers of *Xylella fastidiosa* on PD2 agar as determined by real-time PCR cycle threshold (Ct) values[a]

Spot number[b]	Incubation time on PD2 agar and Ct values[c]				
	4 h	1 day	3 days	5 days	7 days
1	34·85	33·10	32·10	28·86	24·08
6	34·70	34·66	35·25	28·89	27·25
11	35·77	32·59	32·73	29·30	26·77
16	36·16	34·74	39·84	26·94	27·30
21	36·77	33·78	35·88	27·21	27·64
26	38·04	33·52	34·09	30·86	26·86
31	36·13	36·92	35·44	32·52	27·60
Mean Ct value	36·06	34·19	35·05	29·22	26·78

[a]A 1 cm segment from each of three petioles of vine 17-3-2 was used to extract sap and 1 μL used for direct PCR. Controls: direct PCR, sap of all three samples failed to result in a Ct value after 40 cycles; positive control, DNA of *X. fastidiosa* resulted in Ct values of 22–25, depending on the sample; negative water controls failed to result in a Ct value after 40 cycles. Ct value is defined as the PCR cycle number when the signal (fluorescence) rose above the threshold within 40 cycles. Ct values are a mean of two PCR runs.
[b]Five segments (one per incubation time) from one petiole were squeezed and spotted onto separate PD2 agar plates at 35 selected sites; 28 were used for visual detection at day 14.
[c]After 4 h, 1, 3, 5 and 7 days at 28°C, one plate was chosen randomly and the agar surface of sites 1, 6, 11, 16, 26 and 31 removed with a scalpel and used for PCR.

medium, regardless of the time of incubation. The 4 h and 1, 3, 5 and 7 day treatments were all PCR-positive. After 20 days no growth of *X. fastidiosa* was observed on YDC agar, but growth was observed on the PD2 control plate.

Simplified 24 h agar-absorption assay protocol

None of the eight grape samples from Texas was positive by direct PCR. In contrast, two of three samples from both vineyards 1 and 2 were positive by the 24 h agar-absorption assay. Both samples from vineyard 3 were negative by the 24 h agar-absorption assay. Colonies of *X. fastidiosa* were observed on PD2 agar after 14 days for all samples, including those from vineyard 3. All water controls were negative, and all positive controls were positive (Ct values 22–25).

Discussion

The development of real-time PCR has proved to be of considerable interest for the diagnosis of plant diseases (Schaad *et al*., 1999, 2002; Roberts *et al*., 2000; Frederick *et al*., 2000; Weller *et al*., 2000; Weller & Stead, 2002). This technique has the advantage of being less time-consuming and more reliable than classical PCR (Schaad *et al*., 1999; Schaad & Frederick, 2002). A real-time fluorescent PCR protocol for the 1 h detection of *X. fastidiosa* from naturally infected grape with a portable Smart Cycler system has been described (Schaad *et al*., 2002).

This direct real-time PCR method using intact cells works well with dormant tissues, but not so well with actively growing tissue. Also, the sensitivity of this technique is not sufficient to detect samples with low numbers of the bacterium within the xylem system.

In this study the use of a 1 h agar-absorption step and a 5 day enrichment technique (BIO-PCR) significantly improved the effectiveness of detecting *X. fastidiosa*. The simplified 1–24 h absorption protocol allowed for relatively rapid and convenient detection of high numbers of the pathogen, while the 5 day enrichment protocol allowed for detection of samples containing numbers of bacteria below the normal threshold of detection.

The results showed the agar-absorption PCR technique to have much greater sensitivity than direct PCR. Data showing the agar-absorption BIO-PCR method detecting 90% of the infected samples were confirmed by isolation on PD2 agar medium. In contrast, direct PCR detected only 13% of the infected petiole samples. Overall, 16 grapevines from five vineyards and two states were tested by the new agar-absorption PCR protocol and 10 (63%) were positive. In contrast, only one vine was positive by direct PCR. The failure of direct PCR to detect *X. fastidiosa* in the samples was probably caused by PCR inhibitors within the grape sap (Botha *et al*., 2001). It is widely known that some compounds in plant extracts, such as phenolic compounds or polysaccharides, inhibit *Taq* polymerase and therefore inhibit the PCR reaction (Rowhani *et al*., 1993; Minsavage *et al*., 1994). To overcome this problem, different methods have been described, including diluting the plant samples; using a small sample volume (1 μL); extracting DNA; and inactivating PCR inhibitors by adding polyvinylpyrrolidone (PVP) and/or sodium ascorbate (Botha *et al*., 2001) and acid-hydrolysed casein (Mills & Russell, 2003) to the plant sample. However, such measures often fail to increase sensitivity, and the chemicals can be hazardous. The spotting of squeezed sap onto agar medium resulted in a browning of the medium, which was probably the result of phenolic oxidation. The effectiveness of the agar-based PCR may have been the result of absorption of the inhibitors by the medium, leaving bacterial cells on the surface of the agar. This would explain how sufficient PCR amplification was obtained after just 1 h incubation following spotting. The 1 h agar-absorption protocol is a simple and rapid method to overcome PCR inhibitors when high numbers of the pathogen are present. The absorption treatment also works for oak samples. Samples taken from three oak trees showing symptoms of *X. fastidiosa* infection at Fort Detrick in mid-September 2004 were negative by direct PCR, but positive after a 1 h absorption treatment (data not presented).

It is unlikely that multiplication of *X. fastidiosa* occurred within 4 h on the PD2 medium. This was confirmed by the positive results obtained on YDC, a medium on which *X. fastidiosa* does not grow. The Ct values recorded for incubation periods from 4 h to 3 days were similar. Also, maintaining YDC plates for 20 days' incubation did not result in any growth of *X. fastidiosa*. This again suggests that early detection by agar-based PCR was caused by the removal of PCR inhibitors and the presence of an adequate number of cells of *X. fastidiosa*, not by the growth of the bacterium.

These results indicate that high numbers of *X. fastidiosa* can be detected in plant tissues containing PCR inhibitors more reliably after a simple 1–24 h agar-absorption step, just prior to a real-time PCR assay. Furthermore, by combining the agar-absorption step with BIO-PCR, the bacterium can be detected in tissue containing very low numbers in as few as 5–7 days. This is in contrast to the 14–21 days required for classical isolation on agar media. In addition to being very useful for fastidious bacteria, the technique should also be useful for detection of rapidly growing bacteria.

Acknowledgements

We thank the Moroccan–American Commission for Educational and Cultural Exchange in Morocco and the Fulbright Foreign Scholarship Board in Washington, DC, USA for financial support of M.F. Special thanks go to G. Whittenborn, Sunview Vineyards, McFarland, CA and M. Vest, Texas A&M University, College Station, TX for supplying infected grape material with Pierce's disease, and to P. Gaush, USDA-ARS, Fort Detrick, MD for excellent technical assistance.

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